Chapter 8 - Orthotopic Injection of breast cancer cells into the mammary fat pad of mice to study tumor growth

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Short abstract

Cancer is a complex disease that is influenced by the tissue surrounding the tumor as well as local pro- and anti-inflammatory mediators. Therefore, orthotopic injection models, rather than subcutaneous models may be useful to study cancer progression in a manner that better mimics human pathology.

Long abstract

Breast cancer growth can be studied in mice using a plethora of models. Genetic manipulation of breast cancer cells may provide insights into the functions of proteins involved in oncogenic progression or help to discover new tumor suppressors. In addition, injecting cancer cells into mice with different genotypes might provide a better understanding of the importance of the stromal compartment. Many models may be useful to investigate certain aspects of disease progression but do not recapitulate the entire cancerous process. In contrast, breast cancer cells engraftment to the mammary fat pad of mice better recapitulates the location of the disease and presence of the proper stromal compartment and therefore better mimics human cancerous disease. In this article, we describe how to implant breast cancer cells into mice orthotopically and explain how to collect tissues to analyse the tumor milieu and metastasis to distant organs. Using this model, many aspects (growth, angiogenesis, and metastasis) of cancer can be investigated simply by providing a proper environment for tumor cells to grow.

Introduction

Cancer is a very complex disease that has been subject to studies for over centuries. Breast cancer is the most common cancer type; it occurs predominantly in females but may sporadically also occur in males [1]. The disease is mainly caused by the loss of control mechanism governing cell division which in turn leads to an infinite growth of cells in the body. These malfunctions can be caused by several mechanisms: first, healthy cells need growth signals from the surrounding cells in order to proliferate whereas cancer cells make their own growth factors and increase the expression of growth factor receptors thus obtaining a higher proliferative rate [2]; second, cancer cells are less susceptible to anti-proliferative signals [3]; third, to balance the cell number in the body cell death is also required; however, cancer cells escape from programmed cell death, termed apoptosis [4]; fourth, cells adhere to extracellular matrices in order to survive but tumor cells can grow without the need of attachment and show resistance to anoikis [5]; fifth, activation of telomerase circumvents the telomere shortening and prevents the replicative senescence [6]; last but not least, skipping of DNA quality control following mitosis results in altered genetic content [7, 8]. In order to identify oncogenes or tumor suppressors that play a role in this deregulated proliferation, tumor growth experiments in mice are crucial.

Primary tumor growth is generally not the main reason of death. Migration of cancer cells from the primary site to a secondary site, termed metastasis, is the leading cause of death in most cancer patients [9]. Metastasis entails tumor cell invasion, intravasation, travelling through the circulation, avoiding immune attack, extravasation and growth at the secondary site. Epithelial to mesenchymal transition (EMT) is a key process in metastasis and involves a switch in gene expression profiles yielding cells with higher motility and invasiveness, which are pre-requisites for the metastasizing cell [10]. As the cancerous process is the resultant of a combination of various actions, including reciprocal interactions between cancer cells, stromal cells and pro- and anti-inflammatory cells, an *in vitro* approach to cancer often does not provide full insight into the cancerous process. Similarly, anticancer treatments impacting the tumor vasculature can often not be studied *in vitro*, thus the use of *in vivo* approaches is inevitable.

To study breast cancer progression, different experimental methods have been developed. The most widely used model is the subcutaneous injection of breast cancer cells into mice [11]. In this experimental setup, the investigator may introduce a wide range of alterations to a cell line of choice *in vitro* (*i.e* upregulation, downregulation of proteins) and inject the cells under the skin. Although this method is straightforward and the injection process is simple without any need to perform surgery on the mice, the site at which the tumor is injected does not represent the local mammary tumor environment and the absence of this environment may result in breast cancer development that differs from that observed in human pathology. Secondly, genetically engineered mice are used frequently as an *in vivo* tool to study breast cancer progression. In this model, oncogene (*i.e* PyMT, Neu) expression is driven by a mammary tissue specific promoter leading to the formation of spontaneous breast tumors. This experimental setup is useful to study the treatment aspect of the disease by injecting drugs or antibodies while checking tumor size in time [12]. However, breeding these mice with other mouse strains deficient or mutated in a gene of interest might also give insights into the role of different proteins in breast tumor growth [13]. The downside of this model is that it is prone to variation in tumor size and number. Moreover, the level of transgene expression depends on the integration site in the genome and can change from one mouse strain to another [14]. In this model, the expression of the transgene can be achieved by all the cells with epithelial origin whereas in human disease, only a subpopulation of cells express the oncogene or downregulate the tumor suppressor levels [15]. To study metastasis, breast cancer cells may also be injected intravenously (a model termed experimental metastasis) [16]. However, this approach only recapitulates the metastatic process partially; it circumvents

the requirement for tumor cells to invade and intravasate, and starts from the point at which tumor cells are readily present in the circulation.

In our work, we use an orthotopic injection model to study the involvement of genes of interest in breast cancer progression [17]. We overexpress the protein in human breast cancer cells and inject them into the mammary fat pad of NOD/SCID gamma (NSG) mice. This method is advantageous in many ways: it allows very rapid and diverse genetic changes in the injected cell line, it covers the entire process of breast cancer progression from primary tumor growth to metastasis at pathologically relevant sites, and it also provides a good experimental model for studying the impact of therapeutic treatments at early or late stages of the disease. In addition, using this model one can investigate the role of stromal versus cancer cell-derived proteins in disease progression by using genetically modified mice or cells. Although subcutaneous injections are easier to perform, orthotopic models give rise to a more tumorigenic and more metastatic cancer cell population. Thus, results obtained by means of the subcutaneous injections might be either false-negative or false-positive [18, 19] encouraging the use of orthotopic models to study the tumor growth.

Protocol Text:

Animal experiments were approved by the animal welfare committee of the Leiden University Medical Center(LUMC).

1) Preparation of cells, instruments and mice

1.1) A day before operation, shave the NSG mice from the fourth nipple to the midline and weigh the mice to verify that all mice have roughly comparable weights.

1.2) Autoclave a scissor, two tweezers and two straight mosquito forceps **(Fig.1A)** .

1.3) On the day of operation, wash the human breast adenocarcinoma (MDA-MB-231) cells that will be injected once with phosphate buffered saline (PBS), pH 7.4 and trypsinize the cells. Quench the trypsin by adding 10 ml serum-containing DMEM media on top of the cells. Centrifuge the cells at 1200 x g for 7 minutes at room temperature to remove serum by resuspending the cells either in PBS or in media without serum. Centrifuge them again to remove traces of serum completely. Resuspend the cells in PBS or media.

1.4) Count the cells with a hemocytometer and calculate the amount of cells. Use 500,000 cells/mouse in not more than 150 µl. Optional to PBS or media, resuspend the cells in matrigel.

NOTE: Determine the amount of cells depend on the cell type used.

1.5) Place the cells in a sterile microcentrifuge tube and keep the tubes on ice.

1.6) Fill one 50 ml conical centrifuge tube with 35 ml of PBS pH 7.4 and another 50ml conical centrifuge tube with 70% ethanol. Put a cotton swab into each tube.

2) Orthotopic Injection

2.1) Perform the surgery in a biological safety cabinet to prevent contamination. Anesthetize the mouse by subcutaneously injecting anesthetics.. Fix the mouse on a heating pad. Confirm the success of anesthesia by the lack of reaction to toe pinch.

2.2) Apply ophthalmic ointment on the eyes of mice, to prevent the eyes from drying out.

2.3) Clean the shaved area by using the cotton swab dipped into ethanol prepared in step 1.6. Alternatively, one might use ionophore for disinfection.

2.4) Make a small incision between the fourth nipple and the midline with a scissor and make a pocket by inserting the cotton swab moistened with PBS.

2.5) Use a tweezer to expose the mammary fat pad. Observe the fat pad by its white colour.

2.6) Squeeze the fat pad with the other tweezer from its base; by doing this, the fat pad is fully exposed and the injection can be more simply performed **(Fig.1B)**.

2.7) Homogenize the cell mixture by pipetting up and down. Gently aspirate 50 µl of cell suspension into an insulin syringe and inject into the mammary fat pad by holding the needle horizontally. Confirm successful injection by checking for swelling of the fat pad.

2.8) Release the fat pad gently.

2.9) Suture the incision by using mosquito forceps. Make three knots by turning the suture around the mosquito forceps in clockwise, anti-clockwise and clockwise direction.

NOTE: Make sure the knots are tight otherwise mice can open up the sutures.

2.10) After surgery, in order to relieve the pain, inject the analgesic subcutaneously.
2.11) Do not leave the animals unattended until they gain consciousness and maintain sternal recumbence. Place all the animals into different cages until they are fully recovered.

2.12) To maintain sterility, use autoclaved cages and water. Change the cages every three days to keep the atmosphere clean.

3) Harvesting organs for analysis

3.1) At the day of harvest, 8 weeks after the implantation of the cells, fill 15 ml conical centrifuge tubes with 3 ml Bouin's solution for each mouse. In addition, use two 15 ml tubes filled with 5 ml formalin solution per mouse.

3.2) Anesthetize the animals by injecting anesthetics subcutaneously.. Wait until the animal loses toe pinch withdrawal reflex.
3.3) Measure the tumor volume by using a caliper.
3.3) Fix the animal with needles on a base. Make a long, vertical midline incision with scissors. Make two horizontal incisions right below the front leg and above the rear leg. Expose the tumor by pinning the skin to the base.

3.4) Open up the chest cavity with scissors. For blood analysis, withdraw 450 µl blood ,via cardiac puncture, in a microcentrifuge tube containing 50µl of 3.2% sodium citrate.
NOTE: There are several other blood collection techniques commonly used in practice [20] and the technique that needs to be used depends on experimental setup and scientist's choice.

3.5) Dissociate the tumor from the skin using scissors. Snap freeze a part of the tumor in liquid nitrogen for RNA isolation.Place the other part, into the conical centrifuge tube filled with formalin in order to perform immunohistochemistry following paraffin embedding.

3.6) Gently take out the lungs. Place the left lung into Bouin's solution. Keep the lung in solution for 3 days. Observe superficial metastatic foci clearly to naked eye. Optionally, place the right lung into formalin to verify micrometastasis using haematoxylin and eosin (H&E) staining. Although, lung metastasis observed frequently in breast cancer, one might also want to collect bone, liver, brain and spleen to analyse metastasis.

NOTE: The cells at the metastatic area are denser and morphologically different and therefore can be distinguished easily from lung tissue.

3.7) A day after harvest, aspirate the formalin solution from 15 ml tubes and replace with 70% ethanol. Embed the tissues in paraffin and perform immunohistochemistry studies.

3.8) After blood is collected, spin it at 20000 rcf for 10 minutes. Store the plasma samples at -20°C until further use.

NOTE: If blood sample is not required, euthanize the animal according to the animal protocol or as per the institution guidelines.

Representative Results:

Successful application of the "orthotopic breast cancer model" is based on proper injection of cells into the mammary fat pad. Experimental errors such as imprecise inoculation of cells or leakage might lead to variations in tumor size or even the absence of a tumor which leads to the formation of a structure looking similar to a mammary fat pad injected with a control buffer (**Fig. 2A**). The growth rate of the tumor is dependent on the nature of the injected cell line and in general, can be observed through the skin of the mice (**Fig.2B**). Unlike *in vitro* experiments, the growth rate of the tumor cells may not be constant in time *in vivo* (**Fig.2C**). Due to hypoxia and lack of nutrients, necrotic areas may form and these areas will affect the growth rate of the tumor (**Fig.2D**). In addition, the gene of interest might impact a certain phase of cancer progression (early or late), thus depending on the experimental setup, tumor growth might start fast but slow down at the end or vice versa.

Care should be taken during the removal procedure of the tumor; rigorous handling may affect the structure of the tumor and leads to problems in immunohistochemical analysis. The area around the tumor may display large vessels that arise from vascular remodeling.

These vessels play a crucial role in removal of waste products and supply the tumor tissue with nutrients and oxygen thereby facilitating tumor growth (**Fig.2B**). Formation of neovessels (angiogenesis) can be investigated by staining the tumor for neovessel markers (*i.e* CD31, CD34).

Collection of the lungs in Bouin's solution helps to visualize superficial metastatic foci on the lungs (**Fig.3A**). The foci can be distinguished from lung tissue by means of the pale colour and are easy to detect [21]. Metastasis may be expressed as the number of foci formed on the lung surface. However, formation of foci is cell line-dependent; non-aggressive cells give rise to micrometastasis only or no metastasis at all. Micro metastasis can be easily identified with a haematoxylin/ eosin staining on paraffin-embedded lung tissue (**Fig.3C**).

Discussion:

Orthotopic injection of breast cancer cells is a powerful model to study all aspects of cancer growth. Implantation of these cells in the mammary fat pad of the mice should be carefully performed in order to prevent variation in tumor growth. Most importantly, injecting the same amount of cells to each mouse is crucial. To do so, one should trypsinize the cells rigorously without affecting viability of the cells. Non-viable cells should be disregarded during the cell counting and reagents (*i.e* trypan blue) that can help to discriminate between dead and viable cells may be useful for determining the viable cell number. Formation of clumps of cells should be avoided by pipetting the cells up and down as formation of cell clumps cause problems during cell counting and leads to miscalculation of the cell number. Another important aspect that should be taken into consideration is the volume of the cells themselves. As described in the protocol section, cells are pelleted in order to suspend them in PBS or media. Before adding PBS or media, the volume of the cells may be estimated by comparing the pellet with tubes filled with different volumes of media. Subsequently the cell volume may be subtracted from the intended media volume, according to the following formula:

Volume of media that needs to be added = Calculated volume - Cell volume

Reagents that are used in the protocol may affect the outcome of the experiment; therefore the protocol should be adjusted accordingly depending on the research question. For instance, if the experiment aims to elucidate the role of a membrane protein in tumor progression, trypsinization may result in digestion of the membrane protein and cause artefacts [22]. If this is a concern, using buffered EDTA solution or scraping the cells is an alternative to detach the cells. As mentioned above, cells may be resuspended in

media, PBS or matrigel for injection. Among these, matrigel is a convenient option as matrigel polymerizes in the fat pad, thus minimizing cells leaking out of the fatpad. In addition, engraftment in the presence of matrigel may enhance tumor growth and metastatic potential of certain cell lines such as the breast cancer cell line MDA-MB-435 [23], human submandibular carcinoma A253, human epidermoid carcinoma Kb and mouse melanoma B16F10 cells [24]. Please notice that some matrigel preparations contain growth factors that potentially influence the experimental results. However, growth factor-depleted matrigel is available from various suppliers. Additionally, matrigel acts as an extracellular matrix (ECM) and activates integrin subsets. Thus, if the research question involves the role of integrin function in tumor growth, one should use media or PBS as a carrier for breast cancer cells.

In our experimental setup, we injected human breast cancer cells (MDA-MB-231) into the mammary fat pad of NSG mice. The use of immunodeficient mice obviously makes it harder to study the involvement of immunoregulatory genes in cancer development. However, it is important to note that this technique can be also used in mice with intact immune system if the cells that needs to be injected have mouse origin [25]. In addition, MDA-MB-231 cell line is Estrogen Receptor negative (ER-) therefore, the impact of hormones in breast cancer development is missing. Still, our previous work showed that this technique is also feasible for ER+ breast cancer cells such as MCF-7 [17]. Papers utilizing this method to study tumor growth of commonly used breast cancer cell lines are listed below:

Cell Line	Cell Number	Duration of Experiment	Tumor Volume(mm3)	Metastasis	Reference
MCF-7	2x10^6	16 weeks	7	-	[17]
MDA-MB-231	0,5x10^6	8 weeks	375	+	this paper
MDA-MB-231-mfp	0,5x10^6	7 weeks	600	+	[17]
4T1	1x10^6	6 weeks	n/a	+	[25]
MDA-MB-436	2,5x10^6	30 weeks	2000	+	[26]
BT474	2,5x10^6	30 weeks	1700	+	[26]

The procedure itself contains some critical steps as well. Unlike subcutaneous injections, orthotopic injections involve surgical procedures on mice. Hence, surgical tools should be cleaned well and autoclaved. Of note, implantation of human cells in mammary fat pads requires the use of immunodeficient mice (e.g. NOD/SCID). Therefore injections should

preferably be carried out in a biological safety cabinet, using sterile instruments. Moreover, during the injection, the needle should be kept at the right angle with the needle opening facing upwards. Holding the needle in this position reduces leakage and in this manner successful injection can be verified by observing swelling of the mammary fat pad. In addition, when performing surgery, it is crucial to make an incision some distance away from the mammary fat pad in order to avoid interference of the wound healing process with tumor growth. However, the incision should not be made too distant from the mammary fat pad either, as exposing the mammary fat pad might be difficult.

Following the procedure, the animals should be checked regularly. Due to the surgery and exposure to the anaesthetic, mice may experience substantial discomfort or even die. Thus, the first week after the procedure is critical and mice should be monitored carefully. Depending on the growth rate of the implanted cells, tumor volume can be measured up to 4 times a week. The injected cells can also be tagged with a fluorescent protein or luciferase, enabling tracking of primary and metastatic tumor cells, using a light-sensitive camera. Tumors should never be allowed to reach extremely large sizes as ulcerations may occur that damage the tumor tissue. This may hamper proper immunohistochemical analyses of the tumor tissue. Once the tumor is harvested, one might consider mincing the tumor and culturing the dispersed tumor cells to create a new breast cancer cell line to investigate differences between parental and mammay fat pad passed (mfp) cell lines [27].

In conclusion, the orthotopic breast cancer implantation that we outlined in this paper is a very useful tool in order to study the cancer related processes. One can manipulate the genome of the injected cells by either upregulating or downregulating the gene of interest and check its effect on primary tumor growth, angiogenesis or metastasis. This approach is helpful to study proto-oncogenes, tumor supressors or genes that are involved in EMT. In addition, the cell lines can be injected to genetically modified mice to examine the effect of stromal compartment in cancer progression. We strongly encourage the use of the orthotopic injection technique over aforementioned breast cancer models due to its high recapitulation of pathophysiological process.

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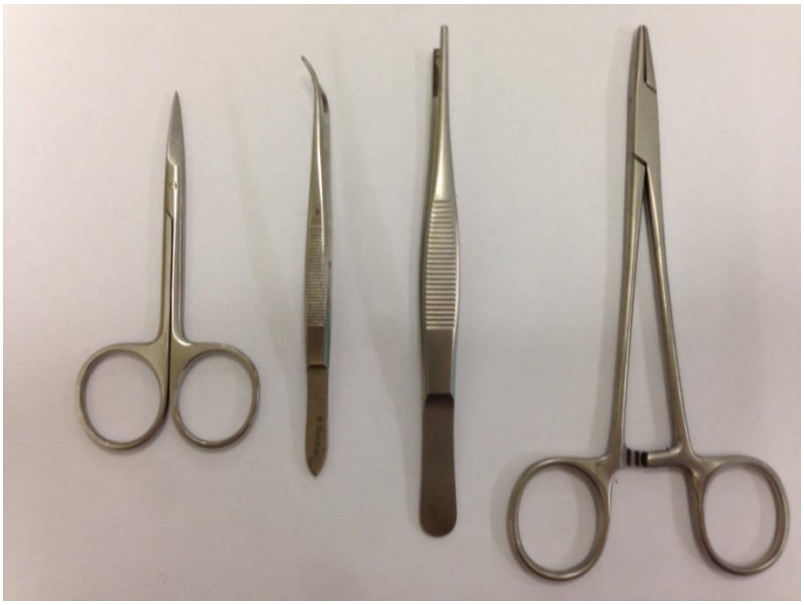
Disclosures:
The authors declare that they have no competing financial interests.

A)



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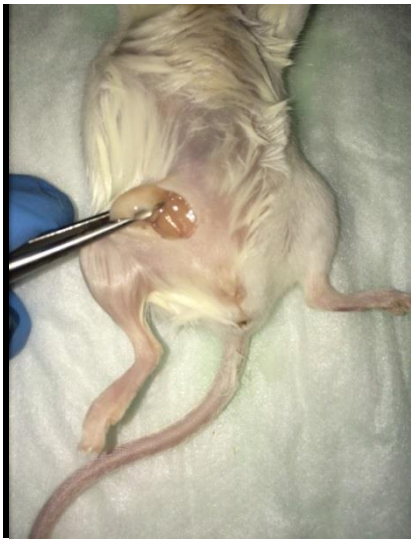


Fig. 1: Materials and Methods. A) Surgical equipments required for the injection of breast cancer cells into the mammary fat pad. B) Representative picture showing the exposure of mammary fat pad.

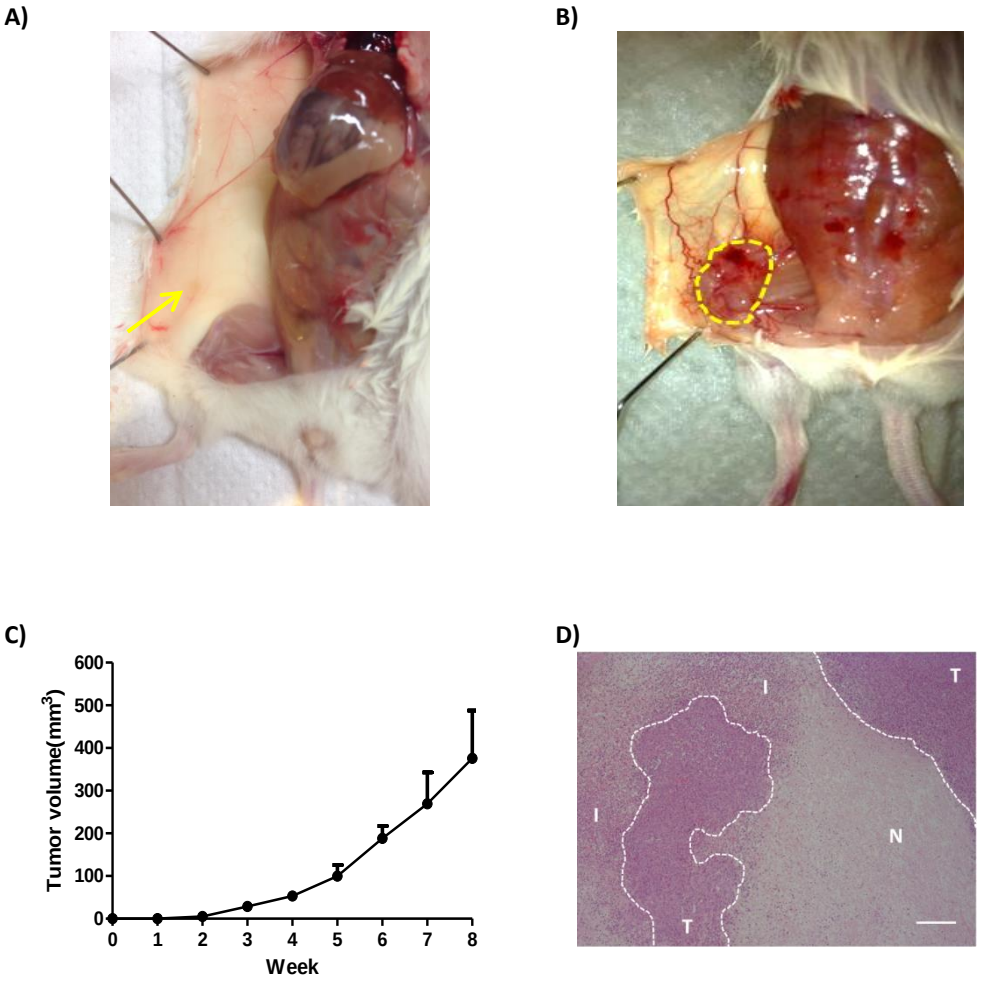
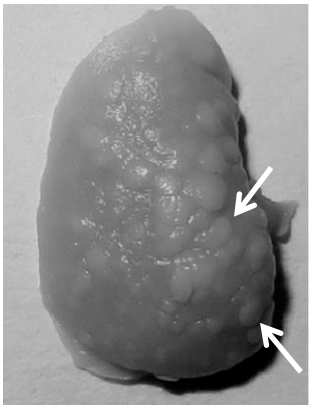


Fig.2: Overview of the orthotopic breast tumor growth in mice. A) Media control or B) Human breast cancer cells (MDA-MB-231) are injected into the mammary fat pad of female NSG mouse. Mice were euthanized and pictures were taken. Tumors are indicated by the dashed circle. The arrow indicates the mammary fat pad. C) Tumor volume was measured with a calliper by using the following formula: Tumor volume = 0.5 x Length x Width x Width. D) Overall morphology of the tumor is analysed with H&E staining. I stands for infiltrated cells, T indicates tumor cells and N stands for necrotic area. (Scale bar=200µm)

A)



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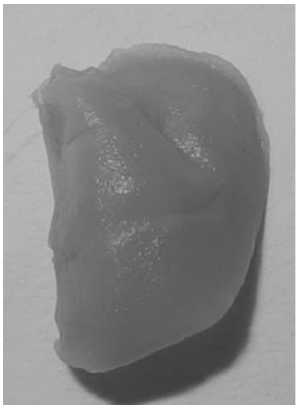

C)

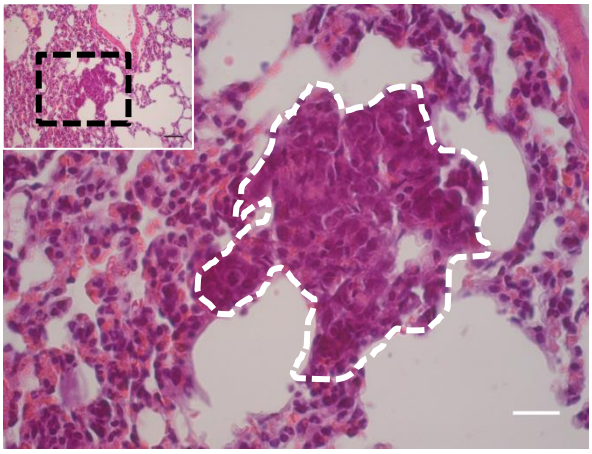

Fig.3: Tumor metastasis to lungs. A) Lungs of mice that were orthotopically injected with tumor cells (MDA-MB-231). Incubation of lungs in Bouin's solution exposes the metastatic foci on lungs. B) Lungs from control mice that underwent orthotopic injection of control media. As can be seen clearly, these control lungs do not show macroscopic metastases. C) H&E staining shows the metastatic region in lung tissue. The insert shows a low magnification overview of the lung tissue containing the metastatic focus. The black dashed rectangle in the insert is represented in the large panel. Tumor cells are indicated by the white dashed line; note the bigger and denser nuclei. (Black scale bar=100µm, white scale bar=20µm)

Name of material/equipment	Company	Catalog number	Comments/Description
Bouin's solution	Sigma-Aldrich	HT10132	Used for investigating the metastasis on lungs
Formalin solution	Sigma-Aldrich	HT501128	Used to fix the tissues
Matrigel, growth factor reduced	Corning	356230	Cells can be resuspended in matrigel for injection
Mosquito forceps	Fine Science Tools	13008-12	Used for stiching
Angled forceps	Electron microscopy sciences	72991-4c	These make the exposure of mammary fat pad easier
Scissors	B Braun Medicals	BC056R	Used to cut open the mice
Straight forceps	B Braun Medicals	BD025R	This is used to open up the skin to expose mammary fat pad
NOD scid gamma mice	Charles River	005557	Experimental animal used for experiment
MDA-MB-231	Sigma-Aldrich	92020424	Experimental cells used for injections
Oculentum simplex	Teva Pharmachemie		Opthalmic ointment used to prevent drying out of eyes
Betadine	Fischer Scientific	19-898-859	Ionophore, used to disinfect the surgical area
Xylazin/Ketamine	Sigma-Aldrich	K1251, K2753	Use injected anesthesia as 10mg/kg and 100mg/kg body weight respectively
Temgesic	Schering-Plough		Use the painkiller as 0,05-0,1mg/kg body weight
DMEM	Life sciences	11995	For trypsin neutralization,use media with serum(FBS:media 1:10 volume); for injection, use media with no serum
Buffered sodium citrate	Aniara	A12-8480-10	Use the volume ratio as citrate:blood; 1:9

Table I. Materials that are used in this paper

Reference List

[1] Weigelt B, Peterse JL, van 't Veer LJ. Breast cancer metastasis: markers and models. Nat Rev Cancer 2005 Aug;5(8):591-602.

[2] Aaronson SA. Growth factors and cancer. Science 1991 Nov 22;254(5035):1146-53.

[3] Fynan TM, Reiss M. Resistance to inhibition of cell growth by transforming growth factor-beta and its role in oncogenesis. Crit Rev Oncog 1993;4(5):493-540.

[4] Lowe SW, Lin AW. Apoptosis in cancer. Carcinogenesis 2000 Mar;21(3):485-95.

[5] Paoli P, Giannoni E, Chiarugi P. Anoikis molecular pathways and its role in cancer progression. Biochim Biophys Acta 2013 Dec;1833(12):3481-98.

[6] Shay JW, Zou Y, Hiyama E, Wright WE. Telomerase and cancer. Hum Mol Genet 2001 Apr;10(7):677-85.

[7] Meek DW. The p53 response to DNA damage. DNA Repair (Amst) 2004 Aug;3(8-9):1049-56.

[8] Meek DW. Tumour suppression by p53: a role for the DNA damage response? Nat Rev Cancer 2009 Oct;9(10):714-23.

[9] Sporn MB. The war on cancer. Lancet 1996 May 18;347(9012):1377-81.

[10] Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. J Clin Invest 2009 Jun;119(6):1420-8.

[11] Ewens A, Mihich E, Ehrke MJ. Distant metastasis from subcutaneously grown E0771 medullary breast adenocarcinoma. Anticancer Res 2005 Nov;25(6B):3905-15.

[12] Brouxhon SM, Kyrkanides S, Teng X, Raja V, O'Banion MK, Clarke R, et al. Monoclonal antibody against the ectodomain of E-cadherin (DECMA-1) suppresses breast carcinogenesis: involvement of the HER/PI3K/Akt/mTOR and IAP pathways. Clin Cancer Res 2013 Jun 15;19(12):3234-46.

[13] Versteeg HH, Schaffner F, Kerver M, Ellies LG, Andrade-Gordon P, Mueller BM, et al. Protease-activated receptor (PAR) 2, but not PAR1, signaling promotes the development of mammary adenocarcinoma in polyoma middle T mice. Cancer Res 2008 Sep 1;68(17):7219-27.

[14] Dobie KW, Lee M, Fantes JA, Graham E, Clark AJ, Springbett A, et al. Variegated transgene expression in mouse mammary gland is determined by the transgene integration locus. Proc Natl Acad Sci U S A 1996 Jun 25;93(13):6659-64.

[15] Wagner KU. Models of breast cancer: quo vadis, animal modeling? Breast Cancer Res 2004;6(1):31-8.

[16] Versteeg HH, Schaffner F, Kerver M, Petersen HH, Ahamed J, Felding-Habermann B, et al. Inhibition of tissue factor signaling suppresses tumor growth. Blood 2008 Jan 1;111(1):190-9.

[17] Kocaturk B, Van den Berg YW, Tieken C, Mieog JS, de Kruijf EM, Engels CC, et al. Alternatively spliced tissue factor promotes breast cancer growth in a beta1 integrin-dependent manner. Proc Natl Acad Sci U S A 2013 Jul 9;110(28):11517-22.

[18] Fidler IJ, Naito S, Pathak S. Orthotopic implantation is essential for the selection, growth and metastasis of human renal cell cancer in nude mice [corrected]. Cancer Metastasis Rev 1990 Sep;9(2):149-65.

[19] Miller FR, Medina D, Heppner GH. Preferential growth of mammary tumors in intact mammary fatpads. Cancer Res 1981 Oct;41(10):3863-7.

[20] Parasuraman S, Raveendran R, Kesavan R. Blood sample collection in small laboratory animals. J Pharmacol Pharmacother 2010 Jul;1(2):87-93.

[21] Mueller BM, Ruf W. Requirement for binding of catalytically active factor VIIa in tissue factor-dependent experimental metastasis. J Clin Invest 1998 Apr 1;101(7):1372-8.

[22] Huang HL, Hsing HW, Lai TC, Chen YW, Lee TR, Chan HT, et al. Trypsin-induced proteome alteration during cell subculture in mammalian cells. J Biomed Sci 2010;17:36.

[23] Bao L, Matsumura Y, Baban D, Sun Y, Tarin D. Effects of inoculation site and Matrigel on growth and metastasis of human breast cancer cells. Br J Cancer 1994 Aug;70(2):228-32.

[24] Fridman R, Kibbey MC, Royce LS, Zain M, Sweeney M, Jicha DL, et al. Enhanced tumor growth of both primary and established human and murine tumor cells in athymic mice after coinjection with Matrigel. J Natl Cancer Inst 1991 Jun 5;83(11):769-74.

[25] Tao K, Fang M, Alroy J, Sahagian GG. Imagable 4T1 model for the study of late stage breast cancer. BMC Cancer 2008;8:228.

[26] Iorns E, Drews-Elger K, Ward TM, Dean S, Clarke J, Berry D, et al. A new mouse model for the study of human breast cancer metastasis. PLoS One 2012;7(10):e47995.

[27] Jessani N, Niessen S, Mueller BM, Cravatt BF. Breast cancer cell lines grown in vivo: what goes in isn't always the same as what comes out. Cell Cycle 2005 Feb;4(2):253-5.